Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells

Cat. #s LVXXX series

User Manual

Check Package Contents for Storage Temperatures

Version 8-05262020

Contents

I. Introduction and Background

A. Purpose of this Manual

This manual provides information describing how to package lentivector expression constructs in pseudoviral particles and use packaged expression constructs for transduction of target cells. Specifically, it provides critical instructions on how to package an HIV-based or FIV-based Lentivector Expression construct in VSV-G pseudotyped viral particles by co-transfecting 293TN Producer Cells with a Lentivector Expression construct and the pPACKH1™ (for HIV-based constructs) or pPACKF1™ (for FIV-based constructs) Packaging Plasmid Mix. Recommendations are also provided for selection and use of HIV-based and FIV-based lentivector systems for transducing a wide range of target cells.

This manual does not include information about construction of expression constructs in lentiviral expression vectors. Information on making constructs using these vectors is available in the user manuals for each of SBI's Lentivector Cloning and Expression Vectors. User manuals, which are provided with each of the Lentivector products, can also be accessed on the SBI website (http://www.systembio.com). Before using the reagents and material supplied with this product, please read the entire user manual.

B. Lentiviral Expression Systems

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (siRNA, cDNA, DNA fragments, antisense, ribozymes, etc.) or reporter constructs in almost any mammalian cell,including non-dividing cells and whole model organisms (Cann, 2000). As with standard plasmid vectors, it is possible to introduce lentiviral constructs in plasmid form into the cells with low-to-medium efficiency and get transient expression of effectors (reporters) using conventional transfection protocols. By packaging the lentiviral expression construct into pseudoviral particles, you can obtain highly efficient transduction (up to 100%), even with the most difficult to transfect cells, such as primary, stem, and differentiated cells.

The expression construct transduced in cells is integrated into genomic DNA and provides stable, long-term expression of siRNA, cDNA or reporter gene. Endogenously expressed siRNA effectors provide long-term silencing of the target gene and allow the researcher to generate cell lines and transgenic organisms with a stable knockdown phenotype for functional studies. Expression of full-length cDNAs from integrated viral constructs is a unique tool to study gain-of-function effect for cellular phenotypes. Stably integrated transcriptional reporter constructs are a novel approach to the study of transcriptional regulation in the natural chromosomal environment and the monitoring of specific signaling pathways. Moreover, lentiviral delivery does not produce the non-

specific cell responses typically associated with chemical transfections or use of an adenoviral delivery system (Gould, 2003, Cann, 2000).

SBI offers both HIV-based and FIV-based lentiviral expression systems. Both systems consist of three main components:

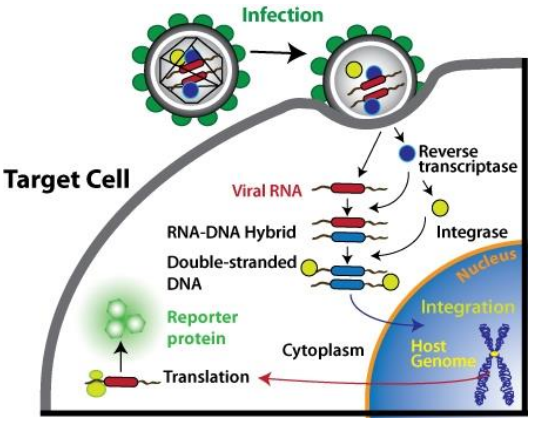
(1) **The lentiviral expression vector** (*e.g.*, shRNA construct in pSIH1-H1-Puro™ or cDNA construct in pCDH lentivector). The lentiviral expression vector contains the genetic elements required for packaging, transduction, stable integration of the viral expression construct into genomic DNA, and expression of the siRNA, cDNA, or reporter.

(2) **The lentiviral packaging plasmids** (*e.g.*, pPACKH1™ Packaging Plasmid mix). The lentiviral packaging plasmids provide all of the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant pseudoviral particles.

(3) **A pseudoviral particle producer cell line** (*e.g.*, 293TN cells). For production of high titer pseudoviral particles, producer cells (*e.g.*, HEK 293 cells) need to be transiently co-transfected with the expression and packaging vectors. Expression constructs packaged in pseudoviral particles are secreted by producer cells in culture media and can be used directly to transduce expression constructs into target cells.

Following transduction into the target cells, this expression construct is reverse transcribed and integrated into the genome of the target cell. After integration, the expression cassette continuously and stably produces high levels of effector or reporter molecules in target cells. Target cells stably expressing the effector molecule can be isolated using the selectable marker contained in the expression vector construct (*e.g.*, puromycin or copGFP). The pseudoviral particles can infect target cells and express effector or reporter molecules but cannot replicate within target cells for two reasons:

1. The viral structural genes are absent
2. The LTRs are designed to be self-inactivating upon transduction



SBI's lentiviral vectors are efficient gene transfer vehicles, as used for research applications, because of their stable integration in non-dividing and dividing cells and long-term transgene expression. Along with our understanding that lentiviral vectors offer solutions for research applications, biosafety concerns have uncovered risks due to insertional mutagenesis, the generation of replication competent lentiviruses and vector mobilization.

Both SBI's HIV-based and FIV-based lentivector systems are designed to maximize their biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) and the CMV promoter (in FIV-based vectors) upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- The number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (*gag, pol, rev*).
- The corresponding proteins are expressed from different plasmids (for HIV-based packaging plasmids) that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, the pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* are present in the packaged viral genome, as they are expressed from separate plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.
- Produced pseudoviral particles will carry only a copy of your expression construct.

The choice of SBI's lentiviral system for experimental studies is driven by functional considerations, including increased productivity and transduction efficiency. The design of SBI's biosafe vectors has benefited researchers allowing them to conduct experimental studies with lower risk. Currently, SBI's vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm. It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and to always follow standard microbiological practices, which include:

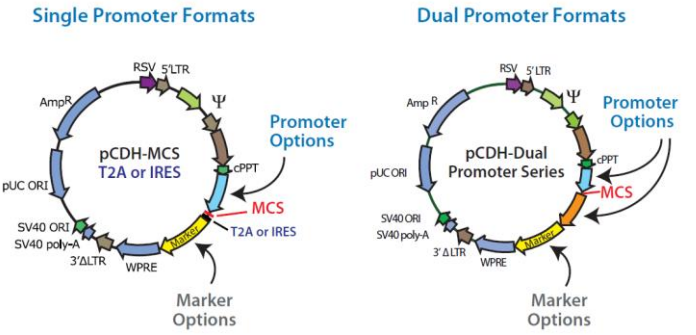
- Wear gloves and a lab coat when handling the lentiviral vectors, pseudoviral particles, or transduced cells.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- Perform all procedures carefully to minimize splashes, spills or the production of aerosols.
- Decontaminate work surfaces at least once a day or after any spill of viable material.
- Decontaminate all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area should be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

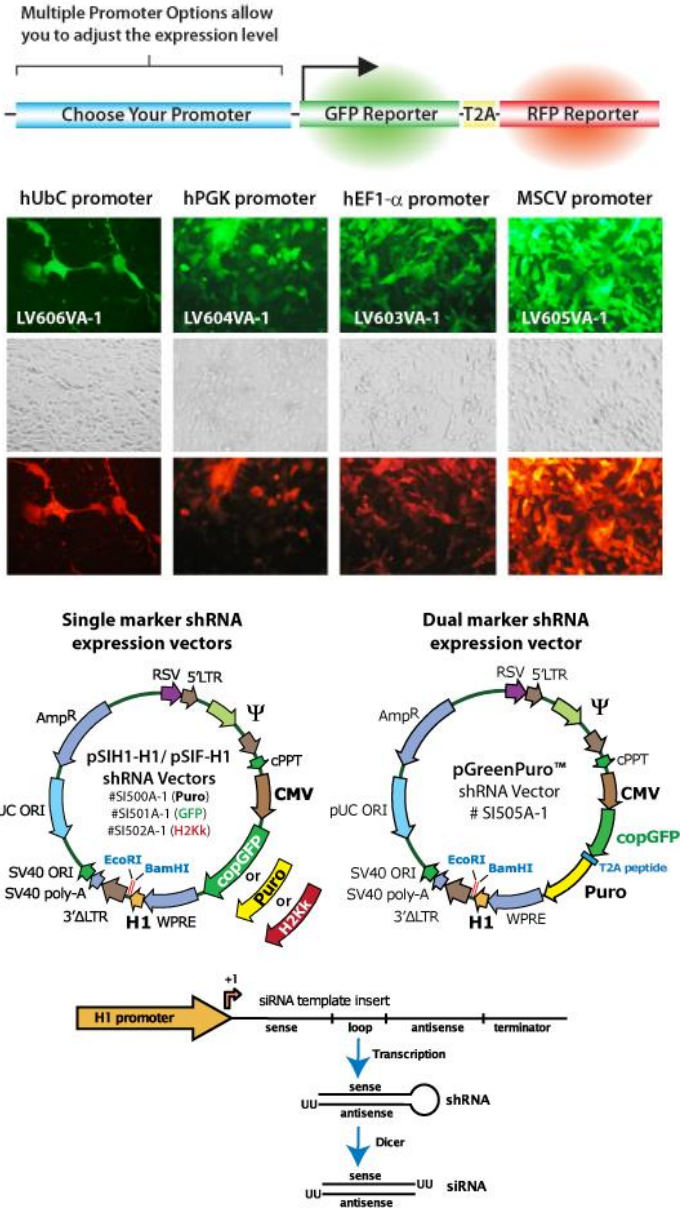
C. SBI's Expression Lentivectors

SBI offers a wide range of HIV-based and FIV-based lentivectors for cloning and expression of siRNA, cDNA and transcriptional reporters. SBI's lentivectors are a third generation of lentivectors developed for gene therapy applications (Poeschla, 2003; Sodroski, J.G., 1997, 1999; Federico, 2003; Heiser, 2004; Machida, 2003). SBI has engineered highly effective and versatile lentivector systems for the expression of shRNA, cDNA, or microRNA sequences as well as transcriptional reporter lentivectors. These lentivectors have similar functional maps and include the following common features:

Hybrid RSV-5'LTR promoter	For HIV-based vectors. Provides a high level of expression of full-length pseudoviral constructs in 293 producer cells.
Hybrid CMV-5'LTR promoter	For FIV-based vectors. Provides a high level of expression of full-length pseudoviral constructs in 293 producer cells
cPPT, GAG, LTRs	Genetic elements necessary for the packaging, transduction, and stable integration of the viral expression construct into genomic DNA
SV40 origin	Provides stable propagation of the lentiviral plasmid in 293 producer cells.
pUC origin	Ensures high copy replication and maintenance of the plasmid in *E.coli* cells
Ampicillin resistance	Used for selection in *E. coli* cells.
WPRE element	Enhances stability and translation of the lentivector-driven transcripts
SV40 polyadenylation signal	Enables efficient termination of transcription and processing of recombinant transcripts.

SBI offers a variety of promoter and reporter options, including GFP, RFP, Puromycin, Hygromycin, Neomycin and Zeocin selection, as well as inducible expression vectors. All SBI lentivectors contain viral stability elements, such as cPPT, WPRE and RRE sequences, for enhanced packaging and infection efficiency.




Multiple Promoter Options allow you to adjust the expression level
Choose Your Promoter
GFP Reporter
-T2A-
RFP Reporter
hUbC promoter
hPGK promoter
hEF1-α promoter
MSCV promoter
LV606VA-1
LV604VA-1
LV603VA-1
LV605VA-1
Single marker shRNA expression vectors
Dual marker shRNA expression vector
RSV 5'LTR
AmpR
Ψ
cPPT
CMV
pSIH1-H1/ pSIF-H1 shRNA Vectors
#SI500A-1 (Puro)
#SI501A-1 (GFP)
#SI502A-1 (H2Kk)
pUC ORI
copGFP
or
Puro
or
H2Kk
SV40 ORI
SV40 poly-A
3'ΔLTR
EcoRI
BamHI
H1
WPRE
pGreenPuro™ shRNA Vector # SI505A-1
T2A peptide
Puro
+1
H1 promoter
siRNA template insert
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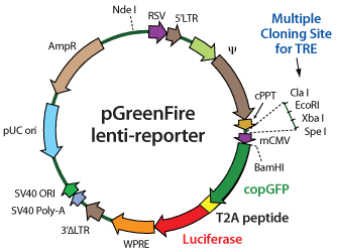


For detailed descriptions of SBI's Expression Lentivectors, please refer to the User Manual for each specific lentivector or visit SBI's web site at http://www.systembio.com.

D. Packaging of Expression Constructs into Pseudoviral Particles

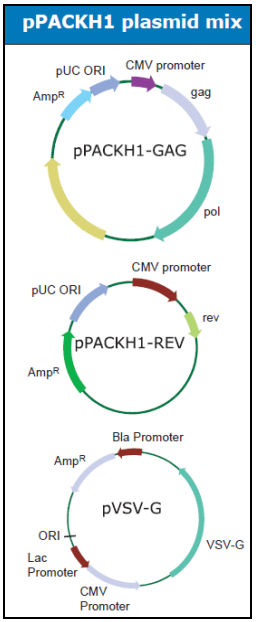
Currently, the most efficient technology for producing a high titer of infectious, replication-incompetent lentiviral particles is based on transient, coordinated expression of a lentiviral expression construct and all necessary packaging proteins delivered into producer cells by simultaneous transfection with lentiviral expression and packaging plasmids. When expressed in packaging cells, the highly-efficient hybrid RSV/5'LTR (or CMV/5'LTR) promoter of the expression construct produces large numbers of the expression construct transcript that contain all of the functional elements (*i.e.*, Psi, RRE, and cPPT) required for efficient packaging. The expression construct transcript is efficiently packaged into VSV-G pseudotyped viral particles with helper proteins produced by the pPACK plasmids. Pseudoviral particles generated by producer cells within 48 – 72 hr can be concentrated, frozen, and used in later experiments.

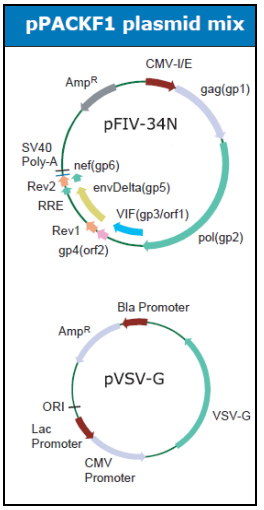
The pPACKH1 Packaging Plasmid Mix consists of an optimized mixture of three plasmids: pPACKH1-GAG, pPACKH1-REV and pVSV-G.

- The pPACKH1-GAG plasmid contains the structural *(gag)*, and replication *(pol)* genes which code for some of the proteins required to produce the lentivirus. The viral *env* gene, which encodes the envelope protein that defines the tropism (*i.e.* the range of infectable cells) is truncated in this construct.
- The pPACKH1-REV plasmid contains the regulatory protein *rev* that is required for HIV replication.
- The pVSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter. VSV-G pseudotyped viral particles mediate viral entry through lipid binding and plasma membrane fusion and can infect both mammalian and non-mammalian cells (Burns, 1993).

The pPACKF1 Packaging Plasmid Mix consists of a combination of two plasmids: pFIV-34N and pVSV-G.

- The pFIV-34N plasmid contains the structural (*gag*), regulatory (*vif*, *gp4, rev, nef*) and replication (*pol*) genes which code for the proteins required to produce the lentivirus. The viral *env* gene, which encodes the envelope protein that defines the tropism (*i.e.*, the range of infectable cells), is deleted in the pFIV-34N construct.
- The pVSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter, thus replacing lentiviral *env* gene. Viral particles, VSV-G protein pseudotyped, mediate viral entry through lipid binding and plasma membrane fusion and can infect both mammalian and non-mammalian cells (Burns, 1993).





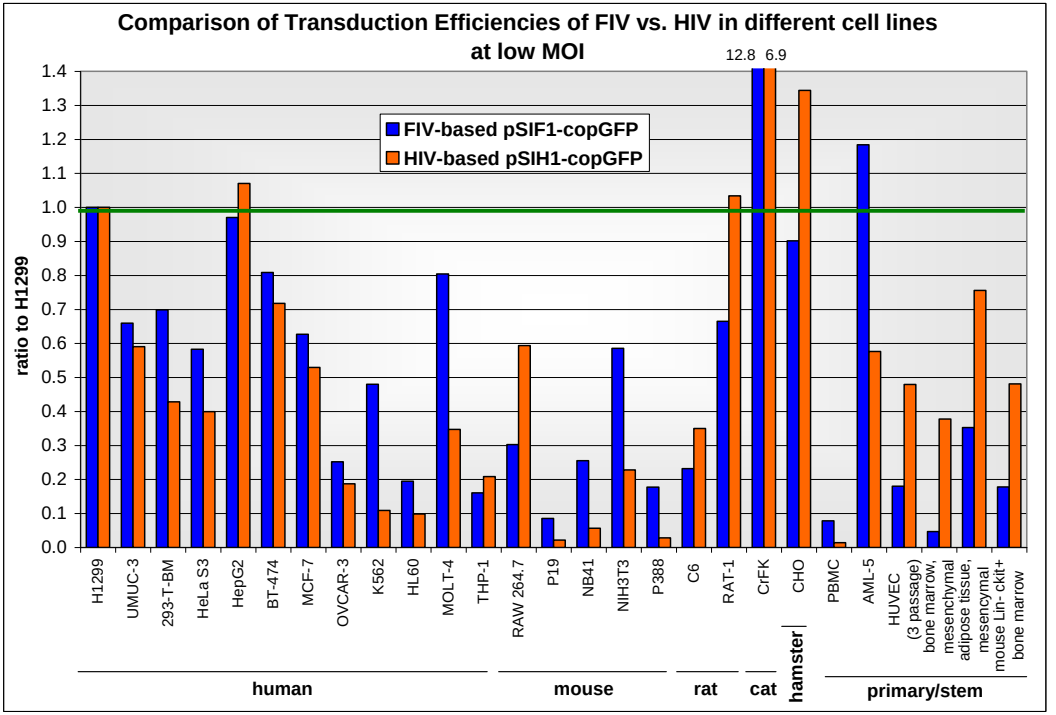
For a more detailed description or sequence information regarding our cloning and packaging lentivectors, visit our web site at http://www.systembio.com.

To facilitate packaging, SBI offers a 293TN producer cell line that was optimized for effective production of a high titer of pseudoviral particles by introduction of the SV40 large T antigen and neomycin resistance gene. The 293TN cell line is a highly transfectable derivative of the HEK293 cell line with constitutive expression of SV40 T-antigen and neomycin resistance gene. It is comparable to the ATCC 293T/17 cell line. 293TN cells may be used in conjunction with the SBI

Lentivector Packaging Kits to produce VSV-G pseudotyped viral particles for transduction of target cell lines.

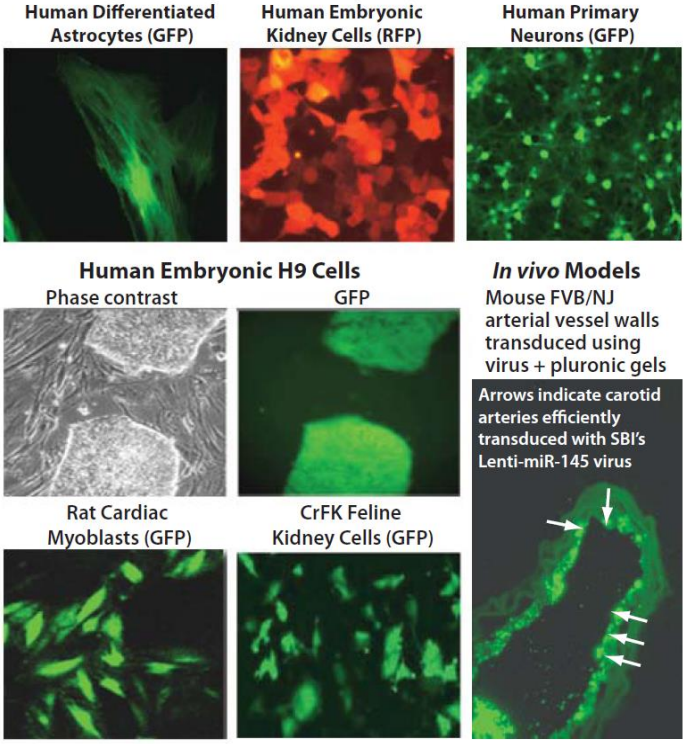
E. Delivery of Packaged Lentivector Constructs into Target Cells

Pantropic VSV-G pseudotyped viral particles containing the RNA copy of the lentivector expression construct can be efficiently used to deliver and stably express effector and reporter sequences in a wide range of mammalian target cells. In order to provide guidelines for the use of lentivector delivery systems, we compared transduction efficiencies of HIV-based and FIV-based vectors in different cell types. The graph below shows a comparison of transduction efficiencies of FIV-based and HIV-based lentivector systems for 27 different cell lines, including primary and stem cells.



These data clearly demonstrate that unlike commonly used cancer cell lines (like H1299, HeLa, HeK295, HepG2, etc.) that can be effectively transduced by lentivectors, some cell types (mouse Lin- ckit+ bone marrow, P19, PBMC, HL60, P388) are more resistant to infection. More efficient transduction of more "resistant" cell types may be possible by using a higher concentration of pseudoviral particles per cell in order to achieve the same MOI, as demonstrated in Appendix A, but not in all cases. It is important to

mention that HIV-based and FIV-based lentivectors have different tropism. For example, the HIV-based system is more effective at infecting stem and primary cells (HUVEC, bone marrow, adipose). The FIV-based system is more effective at infecting several of the tested mouse cell lines (P19, NB41, NIH3T3, P38) and some of the blood cells (MOLT-4, K562, T-cells from AML patient).



Pseudotyped lentiviruses have been successfully used to infect many other cell types, including neuronal, dendritic, endothelial, retinal, pancreatic, hepatic, aortic smooth muscle cells, airway epithelia, skin fibroblasts, macrophages, etc. Lentivectors have been successfully used also for direct *in vivo* delivery and expression of transgenes in muscle, brain, airway epithelium, liver, pancreas, retina, and skin. For a more complete list of cells or tissues, which have been successfully transduced with lentivectors, please see the Reference Section.

F. Essential Lentiviral Products

Lentivectors

cDNA expression lentivectors
http://systembio.com/lentiviral-technology/expression-vectors/cdna/

shRNA Expression lentivectors
http://systembio.com/lentiviral-technology/expression-vectors/shrna/

microRNA Expression lentivectors
http://systembio.com/lentiviral-technology/expression-vectors/microrna/

Transcription Reporter Lentivectors
http://systembio.com/lentiviral-technology/transcription-reporter-vectors/

Expression constructs should be purified with a **QIAGEN Endotoxin-free Plasmid Purification Kit**. The following kit combinations can be used for Midi scale preparation of endotoxin-free DNA:

- QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Maxi Kit, Cat. # 12362
- QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

Please visit the QIAGEN website to download the specialized protocol that is not contained in the user manual: http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

pPACK Lentivector Packaging Kits

The pPACK Packaging Plasmid Mix is an optimized mixture of the packaging plasmids in an amount sufficient for 10 co-transfections with a lentivector expression construct in 10-cm tissue culture plates (or, alternatively, 75 cm^2 flasks). The positive control expression construct with copGFP reporter and shRNA sequence targeting Firefly luciferase is provided in an amount sufficient for 6 control co-transfections with the pPACK packaging plasmid mix.

pPACKH1™ Lentivector Packaging Kit (Cat. # LV500A-1)
for packaging HIV-based lentivector expression constructs

200 µl	pPACKH1 Packaging Plasmid Mix: Mixture of pPACKH1-GAG, pPACKH1-REV, and pVSV-G plasmids (0.5 µg/µl)
20 µl	pSIH1-H1-siLuc-copGFP Positive Control Plasmid (0.5 µg/µl)

pPACKF1™ Lentivector Packaging Kit (Cat. # LV100A-1)
for packaging FIV-based lentivector expression constructs

200 µl pPACKF1 Packaging Plasmid Mix: Mixture of pFIV-34N and pVSV-G plasmids (0.5 µg/µl)
20 µl pSIF1-H1-siLuc-copGFP Positive Control Plasmid (0.5 µg/µl)

The pPACK Plasmid Mix and copGFP Positive Control Plasmid are shipped on dry ice or blue ice and should be stored at -20°C upon receipt. Properly stored plasmids are stable for 12 months from the date received.

Packaged Positive Transduction Controls
http://systembio.com/index.php?id=lentiviral-technology_delivery-systems_positive-transduction-controls/

Packaged VSV-G pseudotyped Positive Transduction Controls are used to estimate and optimize transduction efficiency of lentivector expression constructs and packaged GeneNet siRNA Libraries. The packaged positive controls with copGFP reporter are provided in an amount sufficient to infect ≥2 × 10^5 cells at an MOI of 1. The constructs contain an shRNA targeting Firefly Luciferase.

The Packaged Controls are shipped on dry ice and should be immediately stored at -70°C upon receipt. **Avoid thawing and refreezing of pseudoviral particles!** Each freeze-thaw cycle causes reduction of the titer by 20-30%. Properly stored pseudoviral particles are stable for 6 months from the date received.

293TN Producer Cell Line (Cat. # LV900A-1)
The 293TN Human Kidney cell line is optimized for effective production of a high titer of pseudoviral particles and stably expresses the SV40 large T antigen and neomycin gene products.

The 293TN Cell Line is shipped on dry ice and should be stored at -80°C or liquid nitrogen (gas phase) upon receipt. Properly stored 293TN cells are stable for 1-12 months from the date received, depending on storage conditions.

PureFection Transfection Reagent (SBI. Cat # LV750A-1)
PureFection is a powerful, broadly applicable transfection reagent for effective and reproducible transfections. PureFection reagent self-assembles nanoparticles in the presence of DNA and RNA. These complexes are readily taken up by target cells for efficient gene delivery. PureFection should be stored at 4°C upon receipt.

Peg-It Virus concentration solution (SBI. Cat. # LV810A-1)
PEG-*it*™Virus Precipitation Solution is a formulation of polyethylene glycol optimized for the precipitation of all lentiviral-based particles. It is shipped at room temperature

or on blue ice and should be stored at 4°C upon receipt. Properly stored kits are stable for 1 year from the date received.

Global Ultra Rapid Titering Kit (SBI. Cat # LV961A-1, human and mouse)
The Global UltraRapid Lentiviral Titer Kit is designed to rapidly determine the titers of pseudoviral particles that are generated with SBI's FIV and HIV lentiviral vectors or libraries. It allows users to measure the copy numbers of integrated lentiviral constructs in genomic DNA of transduced target cells.

TransDux virus transduction reagent 200x (SBI. Cat # LV850A-1)
TransDux™ is a unique infection reagent that enables high transduction rates of virus into most cells, even those that are resistant to infections. TransDux is provided as a 200x solution.

G. Additional Required Materials

- **Dulbecco's Modified Eagle's Medium (D-MEM)**
  (high glucose with sodium pyruvate and L-glutamine; Invitrogen, Cat. # 11995073)
- **Fetal Bovine Serum** (Invitrogen, Cat. # 16000036)
- **Puromycin** (Sigma, Cat. # P8833)
- **Penicillin/Streptomycin** (Invitrogen, Cat. # 15070063)
- **Trypsin-EDTA** (Sigma, Cat. # T3924)
- **Tissue Culture Plates and Related Tissue Culture Supplies**
- **Sterile TE Buffer** (10 mM Tris pH 8.0, 0.1 mM EDTA pH 8.0)
- **For PCR Amplification**, Real time PCR System (Recommended: Applied Biosystems 7300 Real time PCR System, Cat# 4351101)

II. Protocol

A. Procedure Outline and General Comments

The figure below outlines the general steps required for packaging of both HIV-based and FIV-based expression constructs, and transduction and expression of the viral expression construct in target cells. To construct a lentiviral expression construct for your experiment, refer to the user manual provided with each specific lentivector.

The lentiviral expression system was designed to simplify all necessary steps in production of pseudoviral particles and transduction of an expression construct into target cells. For general information and background on working with lentiviral technology, we recommend the General Reviews listed in the Reference Section, particularly, Federico, 2003, Cann (2000) and Buchschacher et al. (2000).

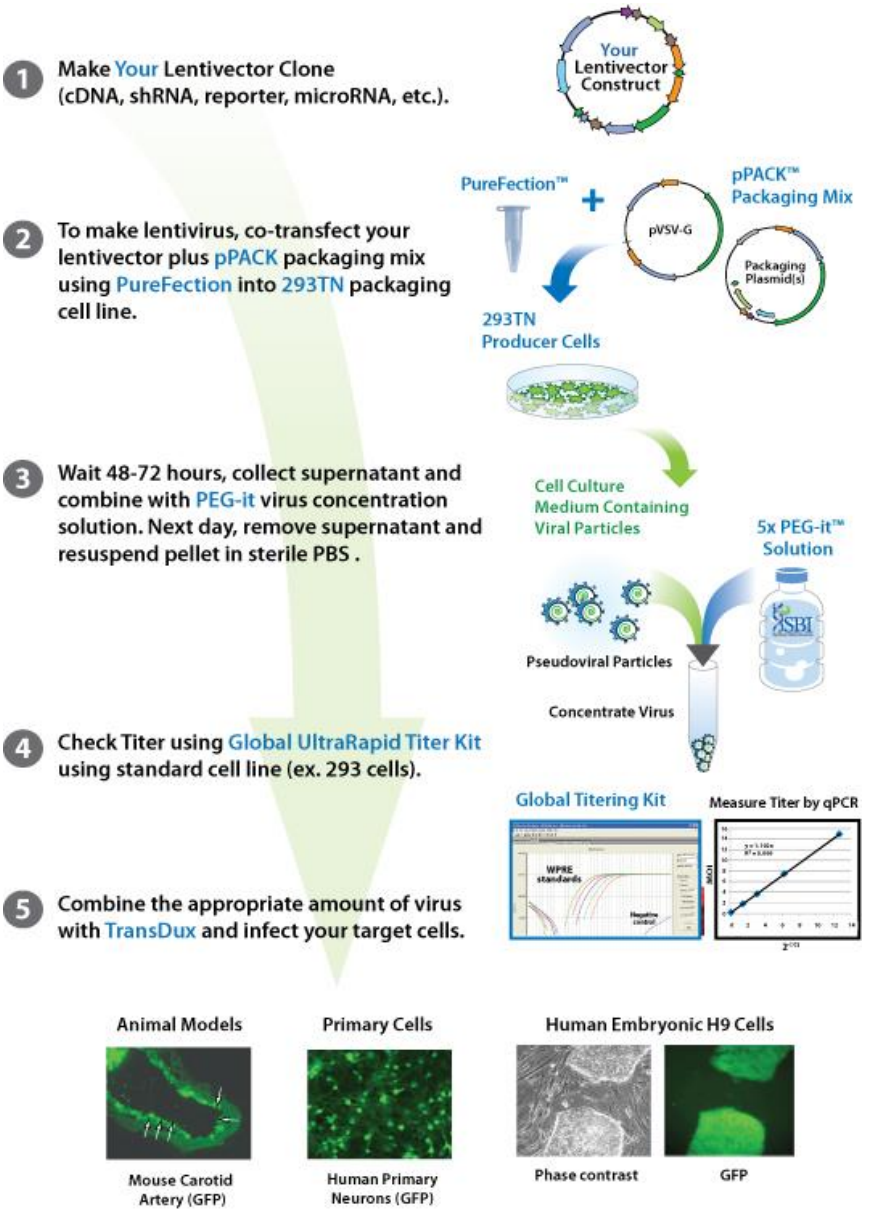
To ensure optimal results, follow these general guidelines during your experiments:

Lentiviral expression construct quality: To generate your specific lentiviral expression construct, refer to the protocol in the user manual provided with the vector. Transfection efficiency significantly depends on the quality of plasmid DNA. We recommend purifying plasmid DNA with a QIAGEN Endotoxin-free Plasmid Kit (see Section I.F). You will need 2.2-3.5 ug μg of lentiviral expression construct in sterile TE buffer with a concentration ranging from 0.2 – 2 μg/μl for each transfection in a 10-cm culture plate.

Maintaining 293TN cell line: The 293TN cell line is a highly transfectable derivative of the HEK293 cell line with constitutive expression of SV40 T-antigen and neomycin resistance gene. The 293TN cells should be grown at 37°C in a humidified chamber with 5% CO_2 in D-MEM medium supplemented with 4 mM L-glutamine, 4.5 g/l glucose, 100 units/ml penicillin G, 100 μg/ml streptomycin (90%), and fetal bovine serum (10%). With a doubling time of less than 24 hours, the 293TN cells should be split every 1 – 2 days when they reach 70 – 80% confluency. For subculturing, detach the cells with 0.25% trypsin, 0.03% EDTA at 37°C, add fresh culture medium, and split at a ratio of 1:3 – 1:5. Alternatively, 293TN cells can be subcultured every 3 to 4 days by splitting cells 1:10 or 1:20, respectively. The cells should never reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase.

pSIH1-H1-siLuc-copGFP or pSIF1-H1-siLuc-copGFP Expression Vector: Included in the pPACK Packaging Kit as a positive control to optimize and troubleshoot your packaging protocol. Specifically, this plasmid should be used as a reference when quantifying viral titer and assaying expression of your construct. If you use a Lentivector without a copGFP reporter and expression of copGFP will not interfere with your

biological assay, you can mix the copGFP construct with your expression construct at a 1:100 ratio and use it as internal positive control at every stage of your experiment.



B. Transfection of 293TN Cells with PureFection™ reagent

To make lentivirus, cotransfect your lentivector construct with the pPACK plasmids into 293TN cells using PureFection reagent. For some viruses, you may need to seed several plates of cells to obtain a high enough titer for transduction of target cells.

1. 18 - 24 hours prior to transfection, seed **7.0 – 8.0 x10^6** 293TN cells per 150 cm^2 cell culture plate* in 20 ml of normal culture medium (without antibiotics) so that the cell density reaches ~60 - 80% confluency at the time of transfection.
2. Add **1-1.6 ml** DMEM (serum free) to an autoclaved 2 ml Eppendorff tube.

   Add **45 µl** pPACKH1 and **5-8 µg** of your lentivector construct (<7 kb use 5 ug, 7 kb-9 kb use 6 ug, 9 kb-10 kb use 7 ug and >10 kb use 8 ug) into the DMEM. Mix by pipetting.
3. Add **55µl** PureFection into the same tube. Vortex for 10 seconds.
4. Incubate DMEM-Plasmid-PureFection mixture at room temperature for 15 minutes.
5. Add DMEM-Plasmid-PureFection mixture drop-wise to the dish, and swirl to disperse evenly throughout the plate.
6. Return the dish to the cell culture incubator at 37°C with 5% CO_2.
7. Change the medium 12-24 hours after transfection (optional).
8. At 48 hours and 72 hours after transfection, collect the medium (which now contains pseudoviral particles) into a 50-ml sterile, capped conical centrifuge tube. Centrifuge at 3000 x *g* for 15 minutes at room temperature to pellet cell debris. Transfer the viral supernatant into a new tube.

Caution: You are working with infectious pseudoviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 safety class.

* If you use 10cm plates, seed 3-4X10^6 cells/ dish in 9 ml of normal culture medium without antibiotics.
- In step 2, 0.8ml of serum free medium should be used for each 10 cm plate.
- In step 3, 20µl of pPACKH1 and 2.2 -3.5 µg of plasmid should be used for each 10 cm plate.
- In step 4, 24 µl of PureFection should be used for each 10cm plate.

C. Concentrate viral particles with PEG-it™ Virus Precipitation Solution

PEG-*it*™ Virus Precipitation Solution provides a simple and highly effective means to concentrate lentiviral particles. PEG-*it* is a formulation of polyethylene glycol optimized for the precipitation of all lentiviral-based particles. **The PEG-*it* Virus Precipitation Solution is a 5x solution.**

1. Transfer supernatant to a sterile vessel and add 1 volume of cold PEG-*it* Virus Precipitation Solution (4ºC) to every 4 volumes of Lentivector-containing supernatant. *(Example: 5ml PEG-it with 20ml viral supernatant).* Precipitation of Lentivector particles from large volumes can be achieved by using the Corning 250 mL polypropylene centrifuge tube (Cat. # 430776), following manufacturer's instructions.
2. Refrigerate overnight (at least 12 hours). Lentivector-containing supernatants mixed with PEG-*it* Virus Precipitation Solution are stable for up to 4-5 days at 4°C.
3. Centrifuge supernatant/PEG-*it* mixture at 1500 × *g* for 30 minutes at 4ºC. After centrifugation, the Lentivector particles may appear as a beige or white pellet at the bottom of the vessel.
4. Transfer supernatant to a fresh tub. Spin down residual PEG-*it* solution by centrifugation at 1500 × *g* for 5 minutes. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated Lentiviral particles in pellet.
5. Resuspend/ combine lentiviral pellets in 1/10 to 1/100 of original volume using cold, sterile Phosphate Buffered Saline (PBS) or DMEM containing 25mM HEPES buffer at 4ºC.
6. Aliquot in cryogenic vials and store at -70°C until ready for use.

D. Determine Pseudoviral titer by real time PCR using SBI's Ultra Rapid Lentiviral Titer Kit

The Global UltraRapid Lentiviral Titer Kit is designed to rapidly determine the titers of pseudoviral particles that are generated with SBI's FIV and HIV lentiviral vectors or libraries. It allows users to measure the copy numbers of integrated lentiviral constructs in genomic DNA of transduced target cells. The kit contains all components necessary for measuring the amounts of endogenous UCR1 DNA element and the pseudo-lentiviral-specific WPRE element that is integrated into the genomes of successfully tranduced cells in each sample. The titer of each sample is then determined by calculating the amount of WPRE element relative to that of Ultra Conserved Region 1 (UCR1) DNA against a standard curve generated with the provided calibration standards. For titering, we use HT1080 cells, as these cells have an average transduction efficiency when compared to 293 or other cells.

We recommend that you titer the pseudovirus-containing supernatant before proceeding with transduction experiments for the following reasons:

- To ensure that pseudoviral stock is viable
- To determine the percentage of target cells which can be transduced with pseudoviral stock
- To control the number of copies of integrated viral constructs per target cell

Below are some key terms used in the protocol:

ifu/ml infectious units/ml	The relative concentration of infection-competent pseudoviral particles
MOI multiplicity of infection	The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. For example, if 1×10^6 cells are to be infected at an MOI of 0.1, then 1×10^5 ifu should be added to the cells
Transduction Efficiency	The average copy number of expression constructs per genome of target cell in the infected (transduced) population

1. For each reaction, you will need 9.5 µl of PCR grade water, 12.5 µl of 2X SYBRTaq Mix, and 1 µl of 25X Primer Mix for either UCR1 or WPRE. Prepare **two** PCR master mixtures (**one for UCR1 and the other for WPRE**) enough for all reactions by multiplying the volume of each ingredient with 2 plus the number of reactions. Combine the required volumes of PCR Grade Water, 2X SYBRTaq Mix, and the Primer Mix in order.
2. Mix contents by inverting the tubes a few times, and spin the tubes briefly in a microcentrifuge.
3. Aliquot 23 µl of the PCR Master Mix into each test tube or well (if you are using a 96-well plate).
4. Add 2 µl of each of the six control DNA calibration standards or the cell lysates from Step A into the test tubes or wells from Step 3. Seal the tubes or plate, and place them in the real time PCR system.
5. Commence thermal cycling using the following program:

   • 50°C for 2 min

   • 95°C for 10 min

   • (95°C for 15 sec; 60°C for 1 min) for 40 cycles

   • Add Dissociation step

6. When the program is complete, check the dissociation curve to make sure there is no significant contamination for WPRE amplification in the negative controls. Then export Ct to an Excel file and calculate the average Ct of UCR1 and WPRE for each standard and sample.

   - Calculate $2^{-\Delta Ct}$, where ΔCt = Average Ct of WPRE – Average Ct of UCR1 of the same standard or sample.
   - Use the Excel software to plot the MOIs* of the standards against the values of $2^{-\Delta Ct}$
   - Use the "add trendline" option of the software to draw the trendline of the standard curve. Set intercept at 0, check the boxes for Display Equation on chart" and "Display R-squared value on chart".
   - Calculate MOI for each of your samples using the equation. For example, if the equation you obtain from your experiment is y = 1.192x, and $2^{-\Delta Ct}$ of one of your samples is 5.1, the MOI of the sample should be 6.08 (i.e. 1.192 multiplied by 5.1).
   - The number of viral particles in your viral suspension (IFU/ml) can then be calculated with the following equation: (MOI of the sample) X (The number of cells in the well upon infection) X 1000 / (µl of viral suspension added to the well for infection).

IMPORTANT: Please be aware that MOIs for each standard provided may vary from lot to lot. Refer to the tube of each standard for MOIs of the particular lot.

E. Transduction of the Packaged Lentivector Expression Construct using TransDux

General considerations:

- **The transduction efficiency of the expression construct** varies significantly for different cells and experimental conditions, including virus concentration, exposure time to virus, and growth area of cells. In order to optimize transduction conditions, we recommend that you use either a titered pseudoviral stock containing the positive control copGFP construct or your packaged expression construct mixed with the copGFP construct. To determine the concentration of pseudoviral particles required to provide the desired multiplicity of infection (MOI) for your target cells, you should do several transductions with different concentrations of packaged pseudoviral particles containing the control plasmid. Results of these test transductions should be used to determine an optimal concentration that yields the optimal percentage of infected cells based on copGFP fluorescence. Note that some cell types, *e.g.* some suspension cultures, may be rather resistant to infection.
- **Expression of the lentiviral construct** can be measured directly at about 48 – 72 hours after transduction ("transient transduction"). Selecting stably transduced cells requires additional time after transduction. For example, puromycin selection usually requires an additional two weeks. The decision to use "transiently

transduced" cells or selected cells depends on the nature of your target cells, biological assay, etc. Some infected, actively dividing cells (*e.g.*, 293, HT1080, HeLa, etc) may express the construct in 100% of cells at an MOI of 1. For these "easy-to-transduce" cells, most biological assays can be performed at 48 – 72 hours after transduction. However, some cells may only express the construct in 10 – 50% of cells, even when transduced with a high concentration of infection-competent pseudoviral particles. For these "difficult-to-transduce" cells, it is probably desirable to select the clones stably expressing the lentivector construct for experimental assays.

- **SBI's Expression Lentivectors** contain a deletion in the 3'LTR which leads to self-inactivation of the lentiviral vector after reverse transcription and integration into genomic DNA. Although more than one copy of a lentiviral construct may be integrated into the genome of a single cell, the lentiviral construct cannot produce infectious viral particles. However, in spite of these safety features, please remember that you are working with transducible pseudoviral particles. Although the particles are replication-incompetent, they are infection-competent, so the expression cassette which they carry will infect, integrate, and express in any mammalian cell type. Please follow the recommended guidelines for working with BSL-2 safety class.

The following protocol uses TransDux™ (SBI). TransDux™ is a unique infection reagent that enables high transduction rates of virus into most cells, even those that are resistant to infections. TransDux is provided as a 200x solution. Use these guidelines as a starting point for determining optimal conditions for your cells and experiments.

Day 1

1. Plate 50,000 cells per well in a 24 well plate in cell culture medium.

Day 2

2. Cells should be between 50 to 70% confluent. Aspirate medium from cells.
3. Combine culture medium with TransDux to a 1X final concentration. (For example, add 2.5 µl of TransDux to 500 µl culture medium and then transfer to each well.)
4. Add virus to each well and swirl to mix. (Optional: Add increasing amounts of virus to different wells at varying MOIs (5, 10 and 20, etc.) to optimize the transduction.

Day 5

5. 72 hours post transduction, the viral genome will be integrated into the host cell genome. Look at the cells for reporter expression if the viral construct has a reporter like GFP.
6. Aspirate off medium. Wash each well with PBS.

7. To establish stabile cell lines, you can now FACs sort for GFP or RFP positive cells. If using an antibiotic selection marker, you can begin your selection procedure.

III. Troubleshooting

A. Low Viral Titer (<10^5 ifu/ml)

1. Poor Transfection Efficiency

293TN Cells have too high or too low density
Plate fewer or more cells in order to have about 50 – 80% confluency at transfection stage.

Lentivector expression construct DNA preparation is of poor quality
Purify plasmid DNA using a QIAGEN Endotoxin-free Plasmid Purification Kit or by phenol/chloroform extraction followed by a CsCl gradient centrifugation.

Plasmid DNA/PureFection™
Optimize the ratios using the guidelines provided in the PureFection™ protocol.

2. Inefficient Production of the pseudovirus

293TN Cells are of poor quality
Optimize growth conditions. Some suggestions are:

- Check growth medium,
- 293TN cells should not be grown for more than 20 passages.
- Check for mycoplasma contamination.
- Make sure the cells have not been overgrown (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).

Pseudoviral supernatant harvested too early or too late
Harvest supernatant every 12 hours starting 24 hours after transfection for 2 – 3 days (24, 36, 48, 60, 72 hours), then titer each batch.

Lentiviral expression construct is too large
The packaging limit for the lentiviral system is 8.5 kb from 5' LTR to 3' dLTR. However, the efficiency of packaging drops significantly at greater than 2 kb of cDNA insert length. For a 3 kb insert, the titers could be 10-fold lower than for a 1 kb insert.

Truncated viral RNA transcript

Re-check the lentivector construct sequence to confirm the absence of a polyadenylation (ATAAA) site between the LTR elements.

B. Inefficient Transduction of Packaged Lentivector Expression Constructs

1. Poor Infection Efficiency

Your stock contains low titer of virus
Optimize infection protocol by using standard pre-packaged pseudoviral stocks of copGFP positive control which can be purchased from SBI (see Appendix F, Related Products).

Volume of infecting supernatant is too high
Keep volume as low as possible to achieve maximal adsorption of viral particles to the cells.

The assay is performed too early
Normally, the maximal expression of integrated provirus is expected to develop by 72 hours after infection. However, some cells display delayed expression. Try the assay at a later time, such as 96 hours.

CMV promoter is not functional in target cells
Replace the CMV promoter with the elongation factor 1 (EF1) promoter in the expression construct.

Target cell line may be difficult to transduce
Check titer with 293TN or H1299 cells. Optimize the transduction protocol. Use a higher MOI.

Loss of pseudoviral titer during storage
Aliquot and store pseudoviral stock at –80°C. Each freeze-thaw cycle drops the titer about 30%. Use a fresh aliquot for transduction.

The cell might methylate some toxic sequences within 10 – 14 days

2. Infection Affects Target Cell Viability

Pseudoviral stock medium affects target cell growth
Dilute the stock medium or concentrate the pseudovirus by centrifugation to minimize the amount of medium added to the target cells. We recommend using SBI's PEG-*it*™ Virus Precipitation Solution (Cat. # LV810A-1).

3. No Expression of Expression Construct

The CMV or H1 promoter is not functional in target cells
We have observed this in primary cells, but the only way to solve this problem is to change the type of target cells or replace the CMV promoter with the EF1 promoter and H1 promoter with the U6 promoter.

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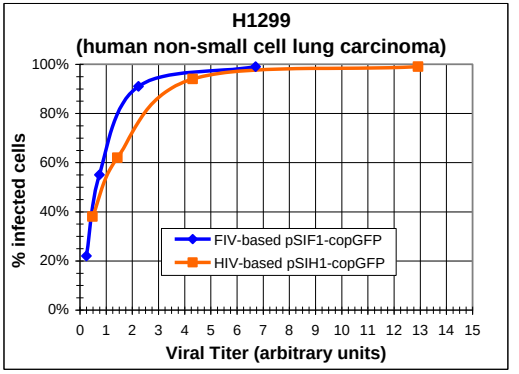
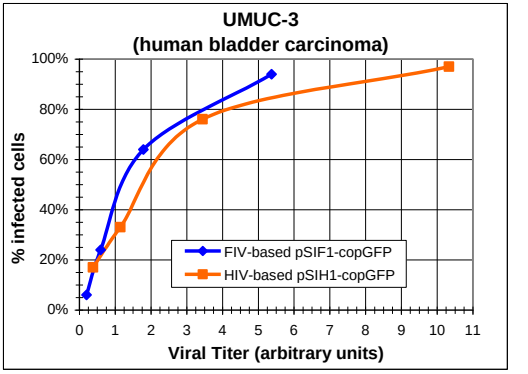
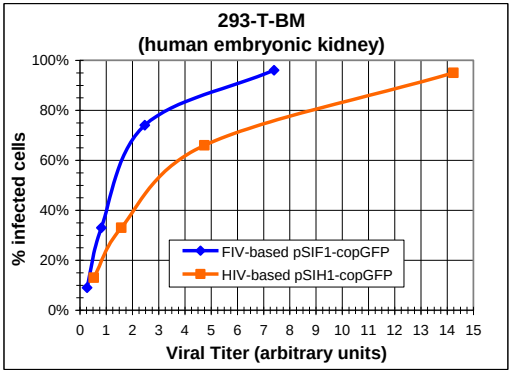
IV. Appendix

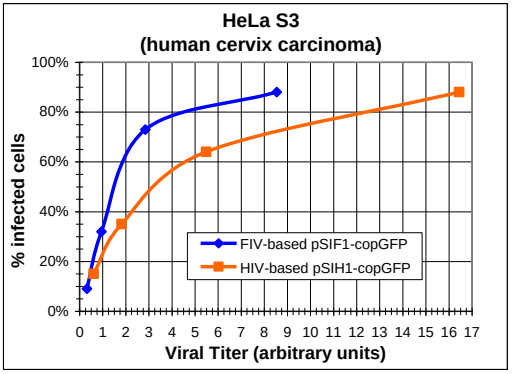
A. Transduction Efficiencies of Different Cell Lines with Increasing Relative Concentration of Viral Particles for FIV-based and HIV-based Lentivectors

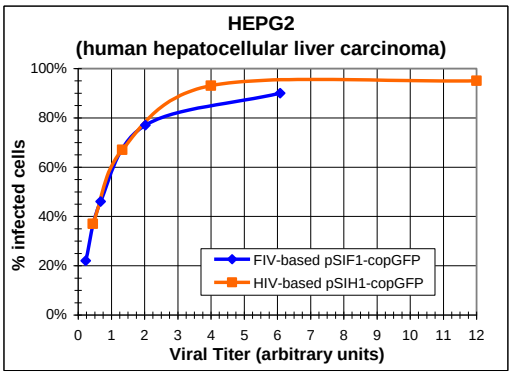
Human Cell Lines

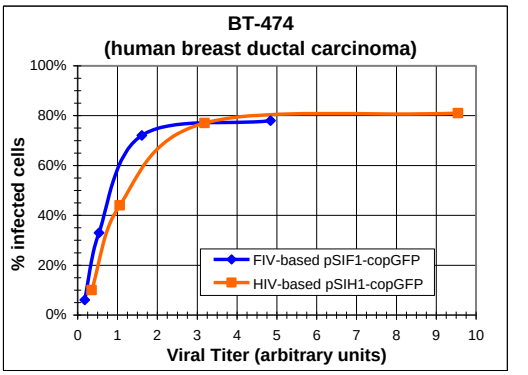












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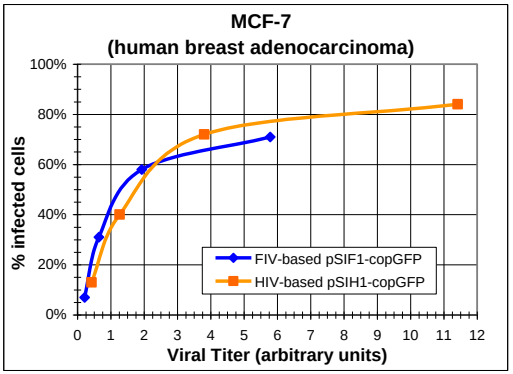
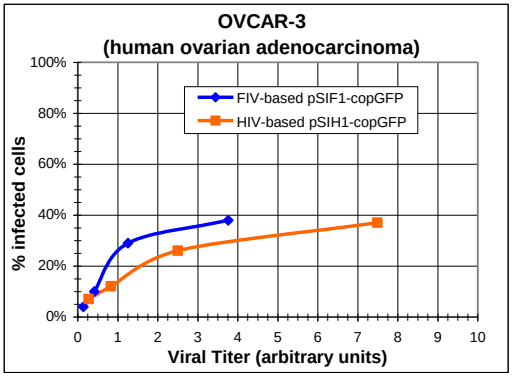
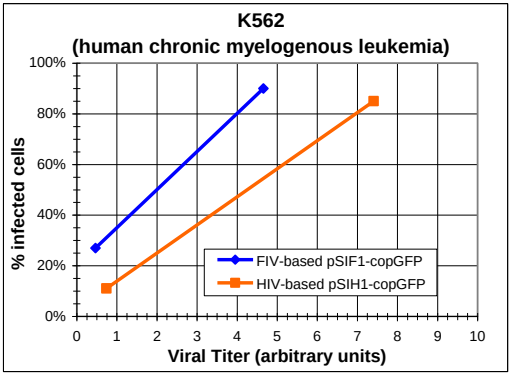
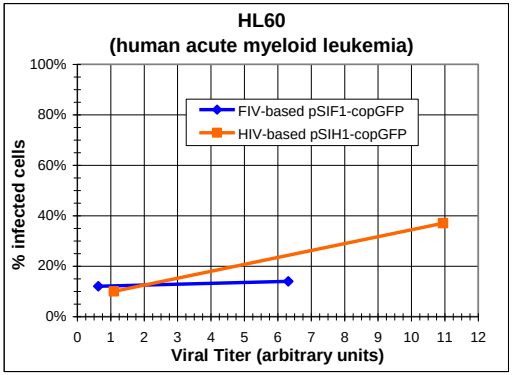
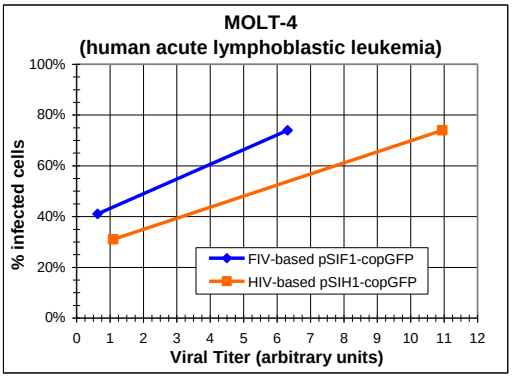
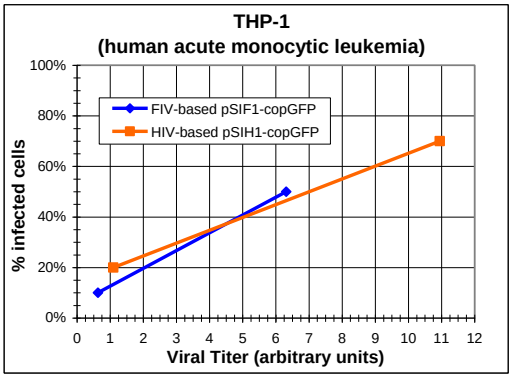




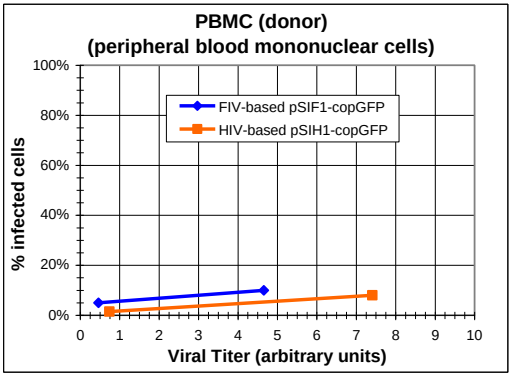


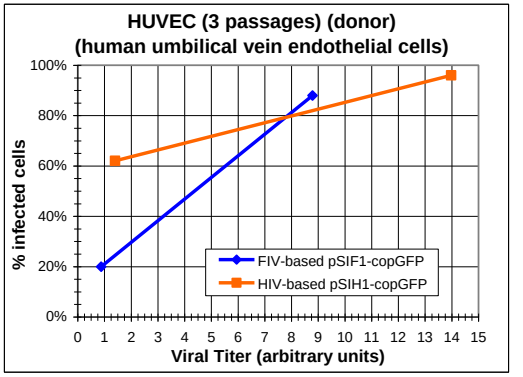


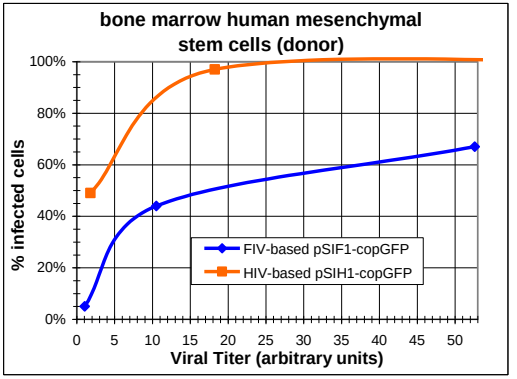


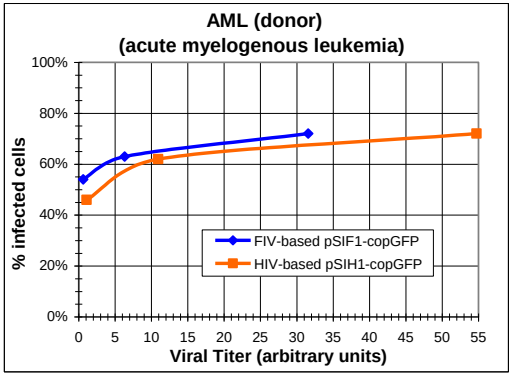


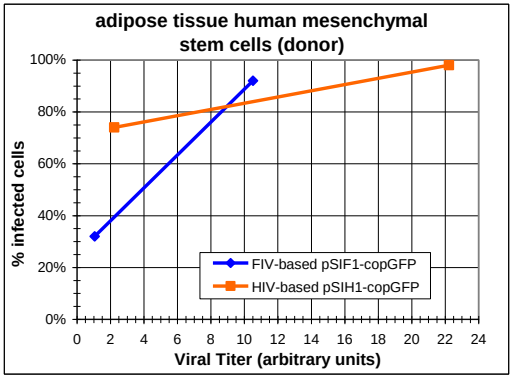
Human Primary/Stem Cell Lines











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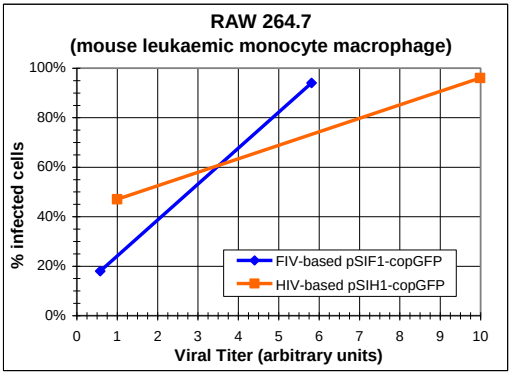
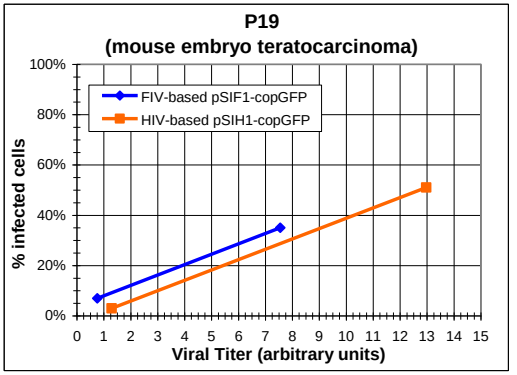
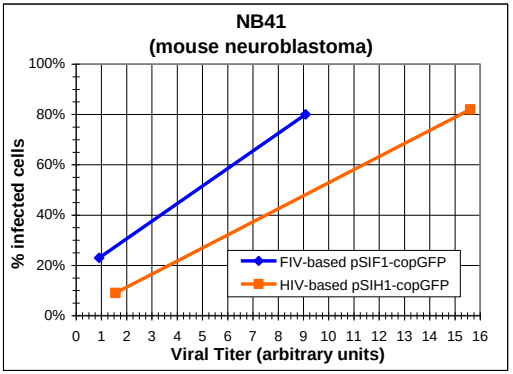
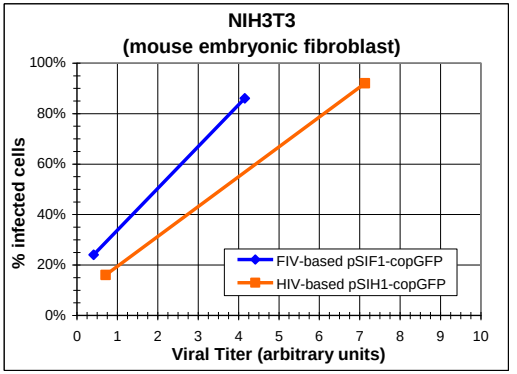
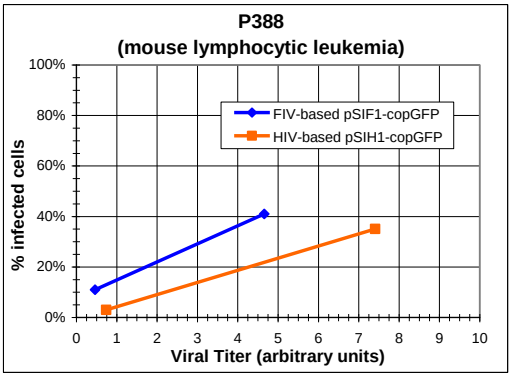
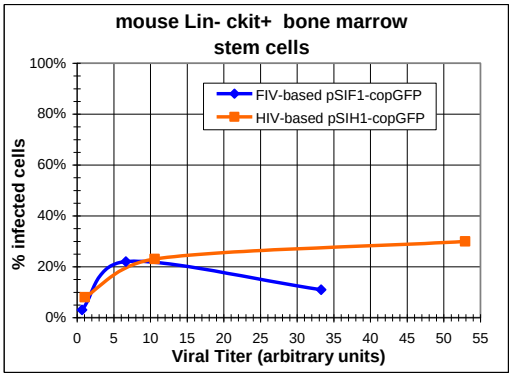




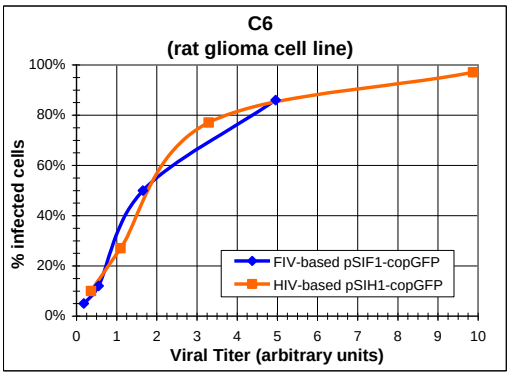


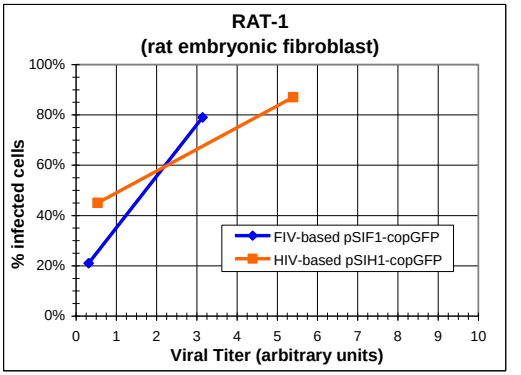




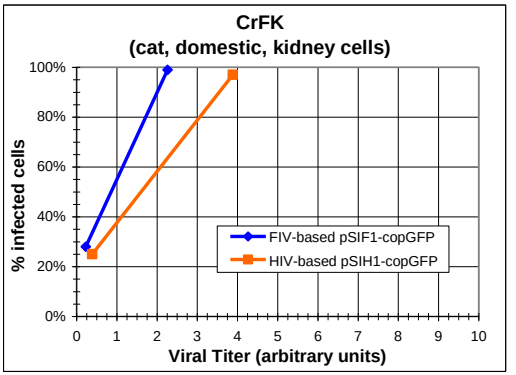


Rat Cell Lines

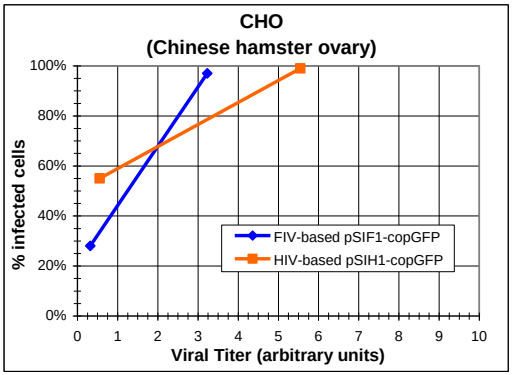




Feline (Cat) Cell Line



Hamster Cell Line



E. Properties of the CopGFP Fluorescent Protein

The pSIF1-H1-siLuc-copGFP, pSIH1-copGFP, and pSIH1-H1-siLuc-copGFP Vectors contain the full-length copGFP gene with optimized human codons for high level of expression of the fluorescent protein from the CMV promoter in mammalian cells. The copGFP marker is a novel natural green monomeric GFP-like protein from copepod (*Pontellina sp.*). The copGFP protein is a non-toxic, non-aggregating protein with fast protein maturation, high stability at a wide range of pH (pH 4 – 12), that does not require any additional cofactors or substrates. The copGFP protein has very bright fluorescence that exceeds at least 1.3 times the brightness of EGFP, the widely used *Aequorea victoria* GFP mutant. The copGFP protein emits green fluorescence with the following characteristics:

emission wavelength max – 502 nm	quantum yield – 0.6
excitation wavelength max – 482 nm	extinction coefficient – 70,000 M^{-1} cm^{-1}

Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of expression constructs into cells.

F. Related Products

- **NF-κB/293/GFP Transcriptional Reporter Cell Line** (Cat. # TR800A-1)
  This human embryonic kidney (HEK)-293-based cell line with a 300-fold NF-κB-dependent activation of a copGFP reporter gene—3 times more sensitive than competitor cell lines—makes analysis of Nuclear Factor kappa B (NF-κB) pathway activation more sensitive and reliable.

- **LentiMag™ Magnetotransduction Kit** (Cat. # LV800A-1)
  A novel, simple, and highly effective approach to increase transduction efficiency with SBI's lentiviral vectors compared to the standard method of Polybrene®-aided transduction.

- **Cloning and Expression Lentivectors** (many)
  Choose from FIV and HIV-based shRNA, miRNA, cDNA, or TRE cloning and expression vectors. For a list of currently available vectors, please visit our website at www.systembio.com.

G. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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1616 North Shoreline Blvd.
Mountain View, CA 94043
Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

V. Licensing and Warranty Statement

Limited Use License

Use of the pPACK Lentivector Packaging Kit, Packaged Positive Transduction Control, or 293TN Producer Cell Line (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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HIV Vector System

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CMV Promoter

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242

CopGFP Control

This product contains a proprietary nucleic acid coding for a proprietary fluorescent protein(s) intended to be used for research purposes only. Any use of the proprietary nucleic acids other than for research use is strictly prohibited. USE IN ANY OTHER APPLICATION REQUIRES A LICENSE FROM EVROGEN. To obtain such a license, please contact Evrogen at license@evrogen.com.

SBI has pending patent applications on various features and components of the Product. For information concerning licenses for commercial use, contact SBI.

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